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REVIEW

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PD-1 and PD-L1 in cancer immunotherapy: clinical implications and future considerations

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ABSTRACT

Programmed death-1 (PD-1) is a cell surface receptor that functions as a T cell checkpoint and plays a central role in regulating T cell exhaustion. Binding of PD-1 to its ligand, programmed death-ligand 1 (PD-L1), activates downstream signaling pathways and inhibits T cell activation. Moreover abnormally high PD-L1 expression on tumor cells and antigen-presenting cells in the tumor microenvironment mediates tumor immune escape, and the development of anti-PD-1/PD-L1 antibodies has recently become a hot topic in cancer immunotherapy. Here, we review the structure of PD-1 and PD-L1, the function of the PD-1/PD-L1 signaling pathway, the application of PD-1 or PD-L1 monoclonal antibodies and future directions for anti-PD-1/PD-L1 antibodies with combination therapies. Cancer immunotherapy using PD-1/PD-L1 immune checkpoint blockade may require more studies, and this approach may be curative for patients with many types of cancer in the future.

1. Introduction

Cancer is the malignant growth of tumors in the human body, and for many types of cancer, there is no cure. In previous decades, the main cancer treatment approaches were surgery, radiotherapy and chemotherapy. However, these treatments cannot distinguish between normal and abnormal cells, which causes unnecessary damage.

More than a century ago, researchers found that the immune system can target tumor cells. As knowledge of immunology increased, T cells were shown to act against tumor cells, with advantages that feature specificity, memory and adaptability[1]. Furthermore, the application of antibodies that block T cell immune regulatory checkpoints is a type of immunotherapy, with breakthrough curative effects[2]. T cells become fully activated through a two-signal model requiring both antigen receptor signaling and CD28 costimulatory signaling. Antibody-mediated inhibition of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) was the first checkpoint protein blockade shown to be effective in cancer immunotherapy; CTLA-4 can translocate to the T cell surface and compete with CD28 for binding to CD80 and CD86, which results in inhibition of T cell proliferation and activation. Other T-cell-intrinsic checkpoints that mediate T cell inhibitory signaling include programmed cell death protein 1 (PD-1), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and lymphocyte-activation gene-3 (LAG-3)[3–5].

In recent years, many researchers are investigating the PD-1/programmed death-ligand 1 (PD-L1) pathway due to its remarkable clinical efficacy, durable response and low toxicity.

The aim of cancer immunotherapies that target PD-1/PD-L1 signaling is to normalize the immune system rather than simply enhancing the function of immune cells in tumor[6]. Regardless, such immune checkpoint therapy only benefits a fraction of patients, and many questions about this approach remain. This review focuses on the PD-1/PD-L1 signaling pathway and progress in its research because antibodies that regulate this pathway are the main immune checkpoint therapies clinically available and because there has been substantial progress in recent years. Accordingly, more information may be learned to provide opportunities to utilize other antitumor immunity antibodies or other methods in an effort to realize their potential for clinical applications.

2. Structure of PD-1 and PD-1 ligands

Human PD-1 (CD279), encoded by the *PDCD1* gene, is a member of the immunoglobulin gene superfamily. This factor was named programmed cell death protein 1, because its expression was shown to be enhanced by apoptotic stimuli in two different cell lines (2B4.11 and LyD9t), and it participates in apoptosis[7]. PD-1 is a type I transmembrane glycoprotein of 50–55 kD that contains a single extracellular IgV domain, a hydrophobic transmembrane domain and a cytoplasmic tail structure domain. The IgV domain consists of 20 amino acids separated from the plasma membrane and exhibits 23% homology with CTLA-4. The cytoplasmic tail contains two tyrosine motifs, an immune receptor tyrosine-based inhibitory motif

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(ITIM) and an immune receptor inhibitory tyrosine-based switch motif (ITSM). Studies have shown that ITSM is necessary to exert the immune suppressive function of PD-1 on active T cells[8].

PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) belonging to the protein B7 family, are the ligands of PD-1[9,10]. PD-L1 and PD-L2 are type I glycoproteins containing IgV and the IgC structure domains, a hydrophobic transmembrane domain and a cytoplasmic tail structure domain. The genes encoding these ligands are both located on chromosome nine, and their sequences are highly conserved. Interaction between PD-1 and PD-L1 occurs in the tumor microenvironment. Briefly, PD-1 is highly expressed on active T cells, and the ligand, PD-L1, is expressed on some types of tumor cells and antigen presenting cells (APCs). Interaction between PD-1 and PD-L1 results in the phosphorylaton of tyrosine residues in the PD-1 cytoplasmic region of the ITSM structure domain, which causes recruitment of Src homology 2 domain-containing protein tyrosine phosphatase-2 (SHP-2). This in turn causes the downstream proteins spleen tyrosine kinase (Syk) and phospholipid inositol-3-kinase (PI3K) to become phosphorylated, which subsequently inhibits downstream signaling and T cell biological functions, including lymphocyte proliferation, cytokine secretion, and cytotoxic T lymphocyte (CTL) cytotoxicity. This interaction results in tumor-specific T cell exhaustion and apoptosis, which enables tumor cells to evade immune surveillance by T cells.

3. Expression and functions of PD-1 and PD-L1 in tumors

Similar to other inhibitory co-receptors, PD-1 is expressed on activated T cells, B cells, monocytes, dendritic cells (DCs), regulatory T cells (Tregs), and natural killer T cells (NKT) [11]. PD-1 expression is defined as a hallmark of T cell exhaustion, which is well-defined in chronic virus infection and cancer[12]. In many types of cancers, PD-1 is expressed on a large proportion of tumor infiltrating lymphocytes (TILs). Among CD4+ TILs, enhanced PD-1 expression is always observed on Treg cells, which may reflect their activation status, whereby the presence of actived Treg cells indicates that the tumor microenvironment (TME) is in an immunosuppressive state. For CD8+ TILs, increased PD-1 expression may reflect an anergic or exhausted T cell state, indicating a loss of CTL function. A recent study found that both mouse and human tumor associated macrophages (TAMs) express PD-1, which decreased their phagocytic capacity against tumor cells; conversely blockade of PD-1/PD-L1 increases phagocytosis and inhibits tumor growth[13].

PD-L1 is commonly upregulated in tumor cells, both in solid tumors and hemangiomas. PD-L1 is also expressed on T cells, B cells, macrophages, DCs, bone marrow-derived mast cells and some non-immune cells[14]. Type 1 and type 2 interferon can increase expression of PD-L1 on tumor cells and APCs[15,16]. In contrast, PD-L2 expression is greatly limited, as it is mainly expressed on activated macrophages and DCs[17]. In addition to tumor cells, PD-L1 is expressed on TAMs, myeloid-derived suppressor cells (MDSCs) and DCs in the TME. Moreover, PD-L1 expression levels on TAMs have been associated with high CD4+ and CD8+ TIL levels in head and neck squamous cell carcinoma[18], and increased PD-L1 expression on MDSCs reportedly maintains their suppressive ability on T cell activation in colon cancer[19]. In multiple myeloma (MM), PD-L1 is expressed on both plasma cell (PC) and DC subpopulations, and PD-L1+ PCs and CD141+ mature DCs inhibit the antitumor T cell response, which is the rationale for using anti-PD-1/PD-L1 antibodies to treat MM patients[20].

The PD-1/PD-L1 pathway plays an important role in auto-immune diseases, virus infection, transplantation immunology, and tumor immunity[1,21–23]. Under normal conditions, the PD-1/PD-L1 pathway induces and maintains peripheral immune tolerance and has a positive effect on preventing excessive tissue inflammation and autoimmune disease. However, with the occurrence and during the development of tumors, the combination of PD-1 and PD-L1 inhibits the host's antitumor immunity, leading to tumor immune escape by 1) inhibiting TIL activation and inducing their apoptosis, 2) inhibiting CTL granular enzyme and perforin production, 3) decreasing the secretion of inflammatory cytokines, such as IFN-γ, IL-2, TNF-α, and promoting the secretion of the immune inhibitory cytokine IL-10, 4) stagnating the T cell cycle, leading to accumulation of cells in G0/G1 phase, and 5) promoting tumor cell epithelial materialization, tumor metastasis and infiltration[24-28](Summarized in Figure 1). Based on the molecular mechanisms of the PD-1/PD-L1 pathway, various types of anti-PD-1/PD-L1 antibodies have been applied to cure tumors, though this treatment has produced successful, durable, and long-lasting responses in only a fraction of patients.

4. Clinical application of PD-1/PD-L1 blockade therapies

4.1 Anti-PD-1 antibodies

Many anti-PD-1 antibodies have been produced to date. Nivolumab (Opdivo), pembrolizumab (Keytruda) and cemiplimab (Libtayo) were approved by the FDA in September 2014, December 2014 and September 2018, respectively (Table 1). Pidilizumab, AMP-224, AMP-514 and PDR001 are still in the experimental phase of development.

4.1.1 Nivolumab

Nivolumab (brand name: Opdivo, also known as BMS-936558 and MDX1106) is a humanized IgG4 anti-PD-1 monoclonal antibody that has a high affinity for PD-1, blocking the binding of PD-1 to its ligand PD-L1. In December 2014, the FDA accelerated the approval of nivolumab for treating unresectable or metastatic melanoma[29], And in March 2015, the FDA approved nivolumab to treatment of metastatic squamous non-small cell lung cancer (NSCLC)[30]. In addition to melanoma and NSCLC, nivolumab has been demonstrated to be effective in a number of other malignancies, including, Hodgkin's lymphoma[31] and hepatocellular carcinoma[32]. The objective response rate (ORR) of nivolumab was found to be 23.7% in patients with NSCLC, with progression-free survival (PFS) of 91
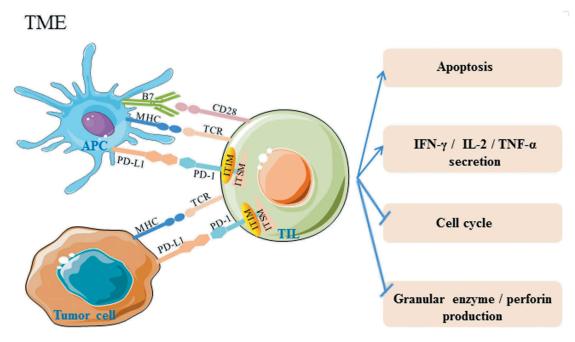
Figure 1. PD-1 and PD-L1 interactions affect TIL function in the tumor microenvironment. In the tumor microenvironment, PD-L1 which can bind to PD-1, is abnormally highly expressed on tumor cells and APCs. Activation of PD-1/PD-L1 signaling can cause phosphorylation of tyrosine residues in the PD-1 cytoplasmic ITIM and ITSM structure domains, decreasing the antitumor activity of TILs by inducing apoptosis, inhibiting granular enzyme and perforin production, decreasing IFN-γ, IL-2, and TNF-α secretion, and stagnating the cell cycle. TCR: T cell receptor; MHC: major histocompatibility complex; APC: antigen presenting cell; ITIM: immune receptor tyrosine-based inhibitory motif; ITSM: immune receptor tyrosine-based switch motif; TIL: tumor-infiltrating lymphocytes. →:activation effect;"⊣ "inhibitory effect.

days[33]. The overall survival (OS) of patients with Hodgkin's lymphoma was approximately 80% at three years, and median the PFS ranged between 12 and 18 months[34].

4.1.2 Pembrolizumab

Pembrolizumab (brand name: Keytruda, also known as MK-3475 and lambrolizumab) is a humanized IgG4 kappa anti-PD-1 antibody with high affinity. In 2014, the FDA approved pembrolizumab to treat metastatic melanoma. A phase I clinical trial showed that pembrolizumab was safe and effective for the treatment of melanoma[35], and a phase II clinical trial showed that the curative effect of pembrolizumab for advanced melanoma was obvious when compared with that of ipilimumab (anti-CTLA-4 antibody)[36]. The ORR of pembrolizumab in patients with advanced melanoma was reported to be 33%[37]. In 2015, advanced NSCLC patient with no previously treatment were using pembrolizumab treatment and got the ORR as 18%[38]. Afterwards, pembrolizumab was approved by the FDA in May 2017 for locally advanced or metastatic urothelial carcinoma. Indeed pembrolizumab improved overall survival compared with the combined performance of vinflunine, docetaxel and paclitaxel in patients with locally advanced or metastatic urothelial carcinoma[39]. Furthermore, early clinical data showed that pembrolizumab also has great potential for the treatment of other tumors. The ORR of pembrolizumab in non-Hodgkin's lymphoma was 53%[40], and in head and neck squamous cell carcinoma was found to be 19%[41].

4.1.3 Cemiplimab

Cemiplimab (brand name: Libtayo) is a high-affinity anti-PD-1 antibody that was the first checkpoint treatment specifically designed for advanced cutaneous squamous cell carcinoma (CSCC); it was very recently approved by the FDA. The result of a phase 1 study of cemiplimab treatment in advanced CSCC patients showed a durable response, as no disease recurrence was found more than 16 months after the treatment[42]. In an expansion phase I study, a 50% response was observed, and the response was durable. Moreover the results of the phase II study showed an objective response of 47%, with adverse events that were similar to other PD-1 inhibitors[43].

4.2 Anti-PD-l1 antibodies

Several anti-PD-L1 monoclonal antibodies are commercially available. Atezolizumab, avelumab and duravulumab were approved by the FDA in September 2014, May 2016, and May 2017, respectively (Table 1). BMS-936559 and CK-301 are still in the experimental phase of development.

4.2.1 Atezolizumab

Atezolizumab (brand name: Tecentriq, also known as MPDL3280A) is a phage-derived human IgG1 monoclonal antibody with an Fc fragment. Atezolizumab blocks PD-L1 on the surface of tumors and exhibits promising antitumor activity. As the Fc fragment of atezolizumab was modified by gene engineering, the antibody-dependent cell-mediated cytotoxicity (ADCC) effect can be avoided[44]. In May 2016, the FDA approved atezolizumab as the first PD-L1 inhibitor for urothelial carcinoma. In addition, this drug has been shown to have significant curative effects in a wide variety of cancers, such as kidney cancer, bladder transitional cell carcinoma,

Table 1. Summary of current anti-PD-1 and anti-PD-L1 antibodies approved by the FDA.

Target	Generic name	Trade name	Class	Company	Phase	Subjects	Groups in the trial	Dose	Tumor type	Endpoints	Reference
PD-1	Nivolumab	Opdivo, BMS-936558, MDX1106	Humanized IgG4	Bristol-Meyers Squibb	III	418	Nivolumab, n = 210	3 mg/kg	Melanoma	OS: 72.9%, PFS: 5.1months, ORR: 40%	[29]
							Dacarbazine, n = 208	1000 mg/m²		OS: 42.1%, PFS: 2.2months, ORR: 13.9%	
					III	272# (352*)	Nivolumab, n = 135	3 mg/kg	Squamous NSCLC	OS: 42%, PFS: 3.5 months, RR: 20%	[30]
							Docetaxel, n = 137	75 mg/m²		OS: 24%, PFS: 2.8 months, RR: 9%	
					II	243	Nivolumab, n = 243	3 mg/kg	Hodgkinlymphoma	PFS: 14.7 months, ORR: 69%	[31]
					I/II	262	Dose-escalation, n = 48	0.1–10mg/kg	HCC	ORR: 15%	[32]
							Dose expansion, n = 214	3mg/kg		ORR: 20%	
					–	38	Nivolumab, n = 38	3mg/kg	NSCLC	PFS: 91days, ORR:23.7%	[33]
	Pembrolizumab	Keytruda, MK-3475, Lambrolizumab	Humanized IgG4	Merck	I	135	No prior ipilimumab, n = 87	10mg/kg or 2mg/kg	Advanced melanoma	ORR: 38%	[35]
							Prior ipilimumab, n = 48	10mg/kg		ORR: 37%	
					Ib	581# (655*)	Ipilimumab-treated, n = 304	2 or 10 mg/kg	Advanced melanoma	ORR: 29%	[37]
							Ipilimumab-naive, n = 277	2 or 10 mg/kg		ORR: 39%	
					I	495	Previously treatment, n = 394	2 or 10 mg/kg	Advanced NSCLC	ORR: 18%	[38]
							Previously no treatment, n = 101	2 or 10 mg/kg		ORR: 24.8%	
					III	542	Pembrolizumab, n = 272	200 mg	Urothelial carcinoma	ORR: 21.1%	[39]
							Control (docetaxel, paclitaxelor, vinflunine), n = 270	175 mg/m², 75 mg/m², 320 mg/m²		ORR: 11.4%	
					Ib	26	Pembrolizumab, n = 26	200 mg	HNSCC	ORR:19%	[41]
	Cemiplimab	Libtayo, REGN2810	Humanized IgG4	Sanofi	I/II	85	Expansion cohorts, n = 26	3mg/kg	Advanced CSCC	ORR: 50%	[43]
							Metastatic-disease cohort, n = 59			ORR: 47%	
PD-L1	Atezolizumab	Tecentriq, MPDL3280A	Humanized IgG1	Roche	Ia	62# (70*)	Clear-cell renal cell carcinoma, n = 62	3 mg/kg, 10mg/kg, 15 mg/kg or 20 mg/kg	RCC	ORR: 15%	[45]
					III	229# (234*)	Atezolizumab, n = 113	1200 mg	10	ORR: 23%	[46]
							Chemotherapy (vinflunine, paclitaxel, docetaxel), n = 116	320mg/m², 175 mg/m², 75 mg/m²		ORR: 22%	
	Avelumab	Bavencio, MSB0010718C	Fully humanized IgG1	Merck, Pfizer	I	108# (115*)	TNBC, PD-L1+, n = 71	10 or 15mg/kg	Breast cancer	ORR: 13% DCR: 27%	[47]
							TNBC, PD-L1−, n = 37			ORR: 5% DCR: 16%	
					II	29# (39*)	Stage IV metastatic MCC, n = 29	10mg/kg	MCC	ORR: 62.1%	[48]
					Ib	184	Stage IIIB or IV NSCLC, n = 184	10mg/kg	NSCLC	ORR: 12%	[49]
	Duravulumab	Imfinzi, MEDI4736	Humanized IgG1	AstraZeneca	II	267	Durvalumab + tremelimumab, n = 133	20mg/kg +1mg/kg	HNSCC	ORR:7.8%	[50]
							Durvalumab, n = 67	10mg/kg		ORR: 9.2%	
							Tremelimumab, n = 67	10mg/kg		ORR: 1.6%	
					III	709# (713*)	Durvalumab, n = 473	10mg/kg	Advanced NSCLC	OS: 66.3%	[51]
							Placebo, n = 236	-		OS: 55.6%	

Abbreviations: NSCLC: non-small cell lung cancer; HCC: hepatocellular carcinoma; HNSCC: head and neck squamous-cell carcinoma; CSCC: cutaneous squamous cell carcinoma; RCC: renal cell carcinoma; TNBC: triple-negative breast cancer; MCC: Merkel cell carcinoma; OS: overall survival; PSF: progression-free survival; ORR: objective response rate; RR: response rate; DCR: disease control rate; #: Number of patients that completed the process of clinical trial; *: Number of patients that enrolled in the clinical trial.

and breast cancer. In the first stage, the safety and validity of 62 patients with renal cell carcinoma were evaluated[45]; the ORR of atezolizumab in the treatment of metastatic bladder transitional cell carcinoma and breast cancer was 26% and 10%, respectively[46,47].

4.2.2 Avelumab

Avelumab (brand name: Bavencio, also known as MSB0010718C) is a fully human anti-PD-L1 IgG1 monoclonal antibody; the drug was announced by Merck and Pfizer in November 2014. By blocking PD-L1, avelumab may reactivate T cells and induce ADCC with its native Fc region. Avelumab also demonstrated an objective response of 62.1% with metastatic Merkel cell carcinoma[48]. Moreover, the ORR of advanced NSCLC was 12%[49].

4.2.3 Duravulumab

Duravulumab (brand name: Imfinzi) is a humanized monoclonal antibody that directly targets PD-L1, blocking PD-L1 and PD-1 interaction on T cells to prevent tumor immune escape and enhance immune responses. An ORR for duravulumab of 9.2% in the treatment of head and neck squamous cell carcinoma (HNSCC) has been reported. In addition, the 6-month progression-free survival rate was 20% in all HNSCC patients, and reached 25% in PD-L1 positive patients[50]. The ORR in NSCLC was 66.3% in the durvalumab group[51].

5. New insights regarding PD-1/PD-L1 blockade for cancer treatment

5.1 Predictive biomarkers

When using anti-PD-1 or PD-L1 antibodies to treat cancer, some patients with low PD-L1 expression might be poor responders. Therefore, to personalize treatments and obtain an optimal treatment effect, biomarkers need to be identified. In patients with thymic epithelial tumor, those with a PD-1-positive microenvironment had a shorter mean estimated survival time than did negative control[52], and in ovarian cancer and hepatocellular carcinoma, patients with high PD-L1 expression had significantly worse outcomes than did patients who had low or lacked PD-L1 expression[53,54]. Moreover, increased soluble PD-L1 (sPD-L1) has been associated with a low 3-year overall survival rate in patients with diffuse large B-cell lymphoma (DLBCL)[55].

Although the FDA has approved a PD-L1 immunohistochemistry (IHC) test as a companion diagnostic for anti-PD-1 antibody treatment in advanced NSCLC, the expression pattern of PD-1 and PD-L1 is not a good predictive biomarker for diagnosing all cancer types because of the following reasons: 1) biopsy specimens from patients may not accurately identify PD-L1 expression; 2) PD-L1 expression levels might differ among patients; 3) biopsy collection times might be too long or involve poor storage conditions, and thus PD-L1 levels might not represent the real PD-L1 status; 4) inappropriate tissue handling or IHC techniques for assessing PD-L1 expression might lead to PD-L1 epitope degradation before fixation; 5) not all antibodies used for PD-L1 are suitable; 6) only membrane PD-L1 is

functional, as it can interact with PD-1 on T cells, and it is unclear whether the amount of cytoplasmic PD-1 expression is associated with cancer progression; and 7) PD-L1 can be expressed by different cells in the TME, and it is also remain unclear whether its expression on different cells is related to cancer progression[14,56]. Given the complexity of T cell regulation, which can be controlled by multiple signaling pathways, other components of the TME may be useful as predictive biomarkers together with PD-1/PD-L1 expression to help doctors select patients for optimal treatment.

5.2 Combination therapy

Currently, an increasing number of researchers are realizing that responses to anti-PD-1/PD-L1 antibody treatment and other immunotherapies are dependent on the TME, which can be distinguished as an immunogenic (hot) TME or a non-immunogenic (cold) TME[1]. Large amounts of TILs and cytokines and high PD-L1 expression occur in hot tumors, whereas cold tumors show almost no PD-L1 expression and no T-cell infiltration. Therefore, some of the reasons why almost combination treatments with anti-PD-1/PD-L1 antibodies may provide better clinical results are as follows: 1) it will create a hot TME, for example, increasing PD-L1 expression or increasing TIL infiltration, which can enhance antitumor activity; 2) it will target other cell types in the TME, such as MDSCs, and provide an additive effect to the entire microenvironment; or 3) it may reduce the dosage or treatment time when compared with a single treatment, which can decrease the side effects of drugs. Overall, there are many combination therapy methods (Figure 2), and these combination therapies have synergistic effects, which can improve the efficacy of therapies in a variety of cancers (Table 2).

5.2.1 Combination with other immune checkpoint antibodies

CTLA-4 and PD-1 inhibit T cell activation in different ways[57]. The first patient to use an immune checkpoint combination was treated with anti-PD-1 and CTLA-4 antibodies in 2009[2]. In metastatic melanoma patients, the response rate for PD-1 and CTLA-4 blockade combination therapy was approximately 60% in phase II and phase III trials when compared with PD-1 blockade alone. Notably, a slightly higher 3-year survival rate was observed, but a higher frequency of toxicity was also found[2]. During 2018, three papers were published on lung cancer treatment with PD-1 plus CTLA-4 blockade. Showing a greater clinical benefit in high-tumor mutational burden patients when compared with low-tumor mutational burden patients, including improved objective responses, durable benefits, and progression-free survival rates[58-60]. Therefore, the tumor mutational burden may be useful as a genomic determinant to identify patients who may benefit from PD-1 and CTLA-4 blockade combination immunotherapy.

Additional clinical trials and pre-clinical tumor model experiments evaluating other checkpoint proteins combined with anti-PD-1/PD-L1 antibodies are also being conducted. LAG-3, TIM-3, T cell immune receptor with Ig and ITIM
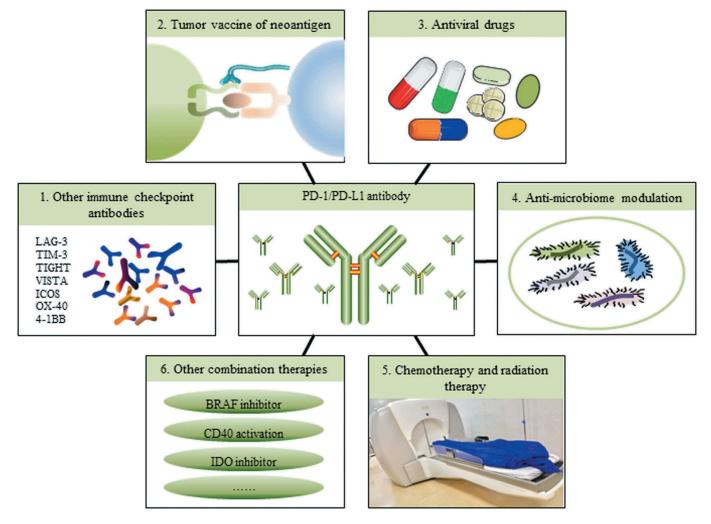
Figure 2. Combination therapies with anti-PD-1/PD-L1 antibodies. Because of the complexity of the tumor microenvironment and the complexity of checkpoint regulation in TILs, only partially successful clinical results of PD-1/PD-L1 blockade have been achieved in certain tumors. Therefore, combination therapies are required. Ongoing studies, such as using PD-1/PD-L1 antibodies combined with other checkpoint antibodies, tumor vaccines, antiviral drugs, anti-microbiome modulation, chemotherapy and radiation therapies, or kinase or enzyme inhibitors are being developed. Combination therapies will be a future direction for cancer patient treatment.

domains (TIGIT), and V-domain Ig suppressor of T-cell activation (VISTA) are all checkpoint proteins in T cells. An investigational study using anti-LAG-3 with and without anti-PD-1 antibodies is currently recruiting patients to test its safety, tolerability and effectiveness for the treatment of solid tumors (NCT01968109, http://www.clinicaltrials.gov). Of note targeting TIM-3, TIGIT and VISTA combined with PD-1 pathway targeting improved antitumor immune responses[61-63]. Inducible T cell costimulator (ICOS), TNF receptor superfamily member 4 (OX-40), and TNF receptor superfamily member 9 (4-1BB) are co-stimulatory molecules in T cells, and antibodies against these molecules combined with anti-PD-1/PD-L1 antibodies resulted in an enhanced immune response in tumor models (NCT02904226, http://www.clinicaltrials.gov)[64,65]. The use of low-dose combination checkpoint inhibition also appears to be an optimal approach for enhancing clinical benefits.

Unfortunately, patients may develop dermatitis, colitis, hepatitis, pancreatitis, pneumonitis and hypophysis after anti-CTLA-4 or anti-PD-1/PD-L1 antibody treatment. In fact, checkpoint combination therapies have notably high toxicity and side effects. For example, most melanoma patients experience immune-related adverse events (irAEs) during anti-PD-1/PD-L1 and anti-CTLA-4 antibody combination therapy[66]. Therefore, further studies investigating the mechanisms of these checkpoint therapies may be required to ameliorate these toxicities.

5.2.2 Combination with neoantigen tumor vaccines
Several studies have shown that genomic information, especially a high mutation burden, helps to determine the response of PD-1/PD-L1 blockade. As it has many mutations that can be recognized as neoantigens, melanoma is a well-known hot tumor, and checkpoint immunotherapy has remarkable clinical effects. Whole-exome sequencing has been applied in NSCLC and mutations in its genomic landscape helped to determine response to anti-PD-1 antibodies[67]. In colon cancer, tumors with mismatch repair deficiency are highly sensitive to anti-PD-1 antibody treatment, and this deficiency indicate many mutant neoantigens[68], which are created by tumor cells because of mutations, and can be recognized by T cells. Although tumor vaccines can be

Table 2. Combination therapies of anti-PD-1 antibodies in clinical trials.

Agent	Target	Tumor type	Phase	Clinical indication being evaluated	Subjects	Groups in the trial and the treatment (dosing regiment and dosage level)		Clinical trials. gov number	Summary of results	Reference
Nivolumab, ipilimumab, chemotherapy (platinum doublet)	PD-1, CTLA-4	Lung Cancer	III	Stage IV or recurrent NSCLC patient not previously treated with chemotherapy and with a high tumor mutational burden (≥10 mutations per megabase).	1739	PD-L1 expression ≥1%	Nivolumab (3mg/kg every 2 wk) + ipilimumab (1mg/kg every 6 wk), n = 396 Nivolumab (240mg every 2 wk), n = 397 Chemotherapy (platinum doublet), n = 396 Nivolumab (3 mg/kg every 2 wk) + ipilimumab (1 mg/kg every 6 wk), n = 187 Nivolumab (360 mg every 3 wk) +chemotherapy (platinum doublet), n = 186 Chemotherapy (platinum doublet), n = 177	NCT02477826	PFS in group of nivolumab plus ipilimumab was longer than group of chemotherapy.	[58]
						PD-L1 expression < 1%				
Nivolumab, ipilimumab	PD-1, CTLA-4	Small-cell lung cancer	I/II	18 years or older patients with an Eastern Cooperative Oncology Group performance status of 0 or 1, irrespective of PD-L1 expression and platinum sensitivity (relapse ≥90 days after chemotherapy) or resistance (relapse <90 days after or during chemotherapy).	401	Nivolumab (3 mg/kg every 2 wk intravenously until disease progression or unacceptable toxicity), n = 245 (TMB-evaluable, n = 133) Nivolumab + ipilimumab (1 mg/kg plus 3 mg/kg every 3 wk intravenously, followed by nivolumab 3 mg/kg every 2 wk until disease progression or unacceptable toxicity), n = 156 (TMB-evaluable, n = 78)	High TMB, n = 47 Medium TMB, n = 44 Low TMB, n = 42 High TMB, n = 26 Medium TMB, n = 25 Low TMB, n = 27	NCT02481830 NCT2538666	Efficacy of nivolumab with or without ipilimumab was enhanced in patients with a high tumor mutational burden.	[59]
Nivolumab, ipilimumab	PD-1, CTLA-4	NSCLC	I	Stage IV non-small cell lung cancer patients.	75	Nivolumab + ipilimumab	High TMB, n = 38 Low TMB, n = 37	NCT01454102	High tumor mutation burden predicted improved objective response, durable benefit, and progression-free survival. Tumor mutation burden was independent of PD-L1 expression.	[60]

Table 2. (Continued).

Agent	Target	Tumor type	Phase	Clinical indication being evaluated	Subjects	Groups in the trial and the treatment (dosing regiment and dosage level)	Clinical trials. gov number	Summary of results	Reference
Nivolumab, ipilimumab	PD-1, CTLA-4	Melanoma	I	Metastatic melanoma patients who had not previously received treatment.	142	Nivolumab (1 mg/kg every 3 wk) + ipilimumab (3 mg/kg every 3 wk), followed by nivolumab (3 mg/kg every 2 wk), n = 95 Ipilimumab (3 mg/kg every 3 wk), n = 47	NCT01927419	The ORR and PFS of the patients with nivolumab combined with ipilimumab treatment were better than those with ipilimumab monotherapy.	[66]

Abbreviations: NSCLC: non-small cell lung cancer; HCC: hepatocellular carcinoma; TMB: tumor mutational burden; ORR: objective response rate; PFS: progression-free survival; wk: weeks.

prepared from cancer cells, parts of cancer cells, or pure tumor antigens, they always contain neoantigens from a tumor. However, questions regarding tumor vaccines from neoantigens have been raised, such as 1) How can neoantigen prediction be optimized？2) What is a good time for vaccine acquisition？3) What is the optimal burden and vaccine injection schedule？4) How do we select patients who will benefit most?[69,70] Among melanoma patients using vaccines that targeted personal tumor neoantigens, those who had recurrent disease were then treated with anti-PD-1 therapy, and complete tumor regression and neoantigen-specific T cell expansion were found after this treatment[71]. These studies provide strong rationale for the further development of this combination approach, though more clinical tests and comprehensive studies identifying mechanisms are required.

5.2.3 Combination with antiviral drugs
Some human cancers are driven by oncogenes from integrated viruses, such as EBV in Burkitt's lymphoma, Hodgkin's lymphoma, gastric carcinoma, HPV in cervical cancer and head and neck cancer, HBV and HCV in hepatocellular carcinoma, and Merkel cell polyomavirus (MCPyV) in Merkel cell carcinoma (MCC) and chronic lymphocytic leukemia (CLL). No clinical trial data for PD-1/PD-L1 blockade combined with antiviral drugs are yet available; however, some preliminary studies indicate promise. MCC is a skin cancer, among which approximately 80% are associated with MCPyV, and high ORRs, prolonged durable responses, and good tolerability were found when using anti-PD-1 therapy[48]. In addition, IHC and cytometry by time-of-flight (CyTOF) have been applied to examine the immune microenvironments in HBV-associated hepatocellular carcinomas, as more PD-1+ Tregs were found in HBV-related HCC than in non-viral-related HCC[72].

5.2.4 Combination with anti-microbiome modulation
In addition to the tumor itself, host factors such as the gastrointestinal microbiome can influence responses to checkpoint immunotherapy. Published in 2018, three notable studies have shown a relationship between healthy gut flora and anti-PD-1 treatment. The authors reported that in melanoma and epithelial cancer, "good" or "favorable" gut bacteria were required for a patient to respond to PD-1 blockade treatment and that antibiotic use could inhibit clinical benefits. Therefore, for anti-PD-1/PD-L1 antibody treatment, therapeutic responses of tumor patients may be improved through host gastrointestinal microbiome modulation[73-75].

5.2.5 Combination with chemotherapy and radiation therapy
Chemotherapy kills tumor cells by inducing DNA damage, cell cycle arrest and ultimately apoptosis, whereas radiation therapy activates the type I IFN pathway in DCs to effectively prime tumor-specific T cells. After more mechanistic studies, it appears that these methods may help turn cold TME into hot TME, as they can create neoantigens of tumors during treatment. Therefore, the response has been encouraging when these two methods have been combined with anti-PD-1/PD-L1 antibodies. In NSCLC, promising results were shown in combination chemotherapy with anti-PD-1/PD-L1 antibodies, including a high overall response and long overall survival but not fewer irAEs[76]. In metastatic melanoma, major tumor regression occurred when radiation and PD-L1 and CTLA-4 dual checkpoint blockade was used[77]. Although these antitumor results are remarkable, a considerable amount of work regarding the dosage, time and sequence is still needed in future clinical trials.

5.2.6 Other combination therapies
There are still many other combination therapies that are under development for application, and most are being tested in melanoma patients. For instance, a BRAF inhibitor used for the treatment of melanoma was combined with anti-PD-1 and anti-CTLA-4 antibodies, and the results showed that BRAF inhibitor therapy may affect subsequent clinical responses to checkpoint immunotherapy[78]. CD40 activation can upregulate APC function, and convert cold tumors into hot tumors. In a pancreatic carcinoma mouse model treated with anti-PD -1 antibodies combined with anti-CD40/chemotherapy, the activity and durability of the response were extended when

compared with anti-CD40/chemotherapy treatment alone[79]. Recently, IDO inhibitor and PD-1 antibody combination therapy exhibited a good antitumor response in hepatocellular carcinoma because checkpoint immunotherapy increased IDO induction, which can cause adaptive resistance in some patients[80]. In another phase 3 trial in advanced melanoma, the same IDO inhibitor plus an anti-PD-1 antibody had no increased benefit compared to the use of the anti-PD-1 antibody alone, indicating that combination therapy may not be necessary at all times; in particular no evidence to date from extensive trials supports the use of combination therapy[81].

6. Conclusion/expert opinion

Recently, cancer immunotherapy targeting PD-1 or PD-L1 has proven effective in causing durable antitumor immune responses with less toxicity in many types of tumors, We believe that PD-1/PD-L1 blockade therapy will be the major cancer immunotherapy method in the next few years, even though there is still much to be learned about this signaling pathway. Key questions remaining to be resolved include: 1) How to select PD-1/PD-L1-positive patient groups? What are their features of these patiens, and what efficient clinical detection method should be used? 2) How can the abundance of tumor infiltrating CD8$^+$T cell be increased in TME? Especially the particular CD8$^+$T cell that display tumor-reactive intratumoral TCR repertoires, but not bystander CD8$^+$TILs that can not kill the tumor cell. 3) What is the mechanism by which PD-1 regulats on CTLs and Tregs, and what is the mechanism by which PD-L1 acts on tumor cells and APC cells in TME? Can we find more efficient inhibitors based on the mechanism? 4) Do we have any good treatment for PD-1/PD-L1- negative patients? Can other therapies or combination therapies with an anti-PD antibody approach be optimal for these patients? With a deeper understanding of personal genomic information, personalized markers in guiding anti-PD therapy alone or with other targets will be critical to achieve clinical results of such therapies, and more work needs to be performed to achieve clarity regarding these key questions. Similar to the tip of an iceberg, PD-1/PD-L1 blockade antitumor immunotherapy opens a new era of cancer treatment, and further work on safety and efficiency will be needed.

Disclosure of potential conflicts of interest

The authors have declared that no competing interest exists.

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